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Expansion of Cytomegalovirus pp65 and IE-1 Specific Cytotoxic T Lymphocytes for Cytomegalovirus-Specific Immunotherapy Following Allogeneic Stem Cell Transplantation

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Adoptive immunotherapy with antigen-specific cytotoxic T lymphocytes (CTLs) has proven effective in restoring cellular immunity to cytomegalovirus (CMV) and preventing viral reactivation after allogeneic stem cell transplantation (SCT). In an effort to develop a cost-effective, relatively rapid method of CMV CTL expansion, we investigated the use of a pool of overlapping CMV peptides. Because the possibility exists of vaccinating CMV-seronegative donors, and these individuals may have T cell responses predominantly against IE-1, commercially available peptide mixes for pp65 as well as IE-1 were used to stimulate CTLs from 10 seropositive donors. Of these 10 donors, 4 responded to pp65 only, 1 did not respond to either pp65 or IE-1, 4 responded to both pp65 and IE-1, and 1 responded to IE-1 only. These CMV- specific T cells included a mixture of $CD4^+$ and $CD8^+$ effectors, and specific cytotoxicity correlated with interferon-γ production. The costs associated with a 28-day maintenance course of intravenous ganciclovir, cidofovir, foscarnet, and valganciclovir, as well as the preparation and shipping a single dose of CTLs, were determined. The price of generating CMV CTLs using this method was comparable to or less expensive than a 28-day maintenance course for these agents, not including the costs associated with drug administration, supportive care, and the treatment of drug-related complications. Considering the relative ease, low cost, and the fact that CTL administration can result in CMV-specific immune reconstitution, this option should be considered for patients with CMV reactivation or for prophylaxis in patients at high risk for infection.

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INTRODUCTION

Although most cytomegalovirus (CMV) infections occurring after allogeneic stem cell transplantation (SCT) will respond to antiviral medications, the use of these agents has been associated with myelosuppression, nephrotoxicity, and impaired immune reconstitution [1,2]. This is particularly problematic for recipients of T cell–depleted SCT, who are at higher risk for CMV infection and disease. There have been several previous reports on treatment with CMV-specific cytotoxic T lymphocytes (CTLs) after SCT, resulting in cellular immune reconstitution and suppression of viremia [3-5]. Methods for growing these CTLs include the use of genetically modified antigen-presenting cells (APCs) to achieve CMV pp65 expression and the pulsing of APCs with CMV peptides or viral lysates [6,7]. Each of these strategies has intrinsic shortcomings. Genetic manipulation of APCs will result in a natural processing and presentation of CMV antigens but is complicated by regulatory issues, high costs, and the time required to qualify viral supernatants and cell therapy products. Depending on the vector and APC used, gene therapy approaches add varying amounts of time for transduction and qualification of APCs, which may be problematic when CTLs are urgently needed [8]. Approaches using individual peptides are limited by our incomplete knowledge of HLA-restricted CMV pp65 epitopes, and CMV T cells expanded using viral lysate may be predominantly $CD4^+$ [4]. Other methods may be limited by the cost of isolating CTLs and the need for specialized equipment, such as selecting CTLs

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based on cytokine production [9], which may preclude widespread implementation. To date, the use of CMV CTLs for treatment or prophylaxis of CMV infection is not routine, and virus-specific immunotherapy is offered at only a few centers that have this as a research interest.

The use of virus-specific CTLs has several potential benefits, including earlier immune reconstitution and avoidance of drug-related complications. Considering the costs of antiviral medications, drug administration, and monitoring and treating complications, the infusion of CMV CTLs may be more cost-effective and directly beneficial to patients. Limitations to immunotherapy strategies targeting a single CMV protein include the fact that in some donors, dominant immune responses may be directed against antigens not used in CTL preparation. CMV pp65 is an important target for CMV-specific CTLs, given the fact that 70% to 90% of all CTLs recognizing pp65 epitopes [10]. Other reports indicate that IE-1–specific T cells also are important in conferring protective immunity to this virus [11,12], including in patients posttransplantation. In addition, IE-1–specific immune responses are stronger and sustained over a longer period after vaccination with the Towne strain of CMV [13], affecting situations in which CTLs are expanded from vaccinated donors. Although to date the major focus in adoptive immunotherapy for CMV has been on pp65-specific immune reconstitution, we decided to investigate whether CTLs with broader antigenic specificity can be generated and, if so, whether the costs associated with cell production can justify the routine use of cellular immunotherapy for CMV. In this work, we demonstrate that CMV pp65 and IE-1–specific CTLs can be reliably expanded from most normal donors, and that infusing these CTLs can be a cost-effective strategy for treating patients with CMV reactivation.

METHODS

Cell Culture

Donor blood specimens were collected under a protocol approved by the Penn State Hershey Medical Center Human Subject Protection Office. Human leukocyte antigen (HLA) testing was performed serologically for HLA-A, -B, and -DR at the Penn State Hershey Histocompatibility Laboratory. Between 60 and 80 mL of peripheral blood was collected from each donors, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. Between 50 and 100 $\times$ 10^6 PBMCs were placed in 15 mL of RPMI (Gibco, Chicago, IL) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) for 1 hour in T150 flasks (Corning, Corning, NY). Nonadherent peripheral blood lymphocytes (PBLs) were removed and resuspended at 2 $\times$ 10^6/mL in RPMI 1640/10% FBS, and adherent cells were removed with a cell scraper (Corning). These adherent cells were then washed in RPMI 1640 and placed at 10 $\times$ 10^6 in 0.5 mL of RPMI in 50-mL conical centrifuge tubes, then pulsed with either CMV pp65, IE-1, or both peptides simultaneously. The CMV pp65 and IE-1 peptide mixes (JPT Peptide Technologies, Berlin) consisted of 138 and 120 overlapping 15mers, respectively. Each peptide were suspended in 40 μL of DMSO (Sigma Aldrich, St. Louis, MO) in accordance with the manufacturer's instructions. Then 3 μL (0.7 mg/mL) of the peptide suspension was added to 10 to 20 $\times$ 10^6 adherent cells, and the capped centrifuge tubes were incubated at room temperature for 2 hours, then washed/centrifuged in RPMI 1640 3 times. PBLs were plated with peptide-pulsed adherent cells at a responder:stimulator ratio of 10:1 in 24-well plates (Corning), 2 mL per well. CTLs resulting from this method were analyzed by chromium release assays and flow cytometry for intracellular cytokine production.

Chromium Release Assays

Targets for chromium release assays (CRAs) included autologous and allogeneic B cell blasts (BBs; used as a negative control) and BBs pulsed with the pp65 or IE-1 peptide mixes. To determine whether these effector cells recognized naturally processed and presented pp65 and IE-1 epitopes, we also infected BBs with vaccinia encoding pp65 and IE-1 (vacc-pp65, provided by Dr William Britt, University of Alabama Birmingham, and vacc-IE-1, provided by Dr Don Diamond, City of Hope). BBs were cultured from donor PBMCs as described previously [14]. Targets were labeled overnight with ^{51}Cr (100 μCi/10^6 cells; PerkinElmer Life and Analytical Science, Boston, MA), washed in PBS, and dispensed in triplicate into 96-well V-bottom plates (ICN, Costa Mesa, CA) at 4 $\times$ 10^3 cells/well, as described previously [6]. CTLs were added at a responder:target ratio of 10:1, and after pelleting and incubation for 4 hours, the supernatant was analyzed in a gamma counter. Spontaneous and total releases for each target were used to calculate percent specific release by the following formula: % specific release = (experimental cpm − spontaneous cpm)/ (total cpm − spontaneous cpm).

Intracellular Cytokine Staining

Flow cytometry for interferon (IFN)-γ production was performed with a FACScan (BD Biosciences, San Jose, CA) to detect pp65 and IE-1 -specific T cells. Multiple-color staining of immunophenotypic markers, both surface and intracellular, was performed as described previously [15]. Surface markers of CTLs were determined by staining with directly

conjugated monoclonal antibodies (mAb) specific for CD3, CD4, CD8, and CD56 (BD Biosciences). Cultured T cells were incubated with equal numbers of stimulators, including autologous BBs pulsed with pp65 and IE-1 overlapping peptides, autologous and allogeneic BBs in RPMI 1640 with 10% FCS, and in the presence of 10 μg/ml brefeldin A (Sigma) at 37°C for 5 hours. After incubation in FACS permeabilization buffer (BD Biosciences) for 10 minutes, cells were aliquoted and stained with the following labeled antibodies: CD4-FITC, CD8-peridinin chlorophyll protein, and IFN-γ APC (BD Biosciences).

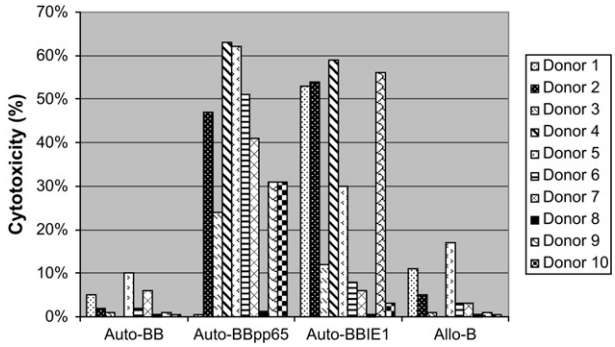

Figure 1. Cytotoxicity of CTLs. PBMCs were pulsed with pooled CMV pp65 and IE-1 peptides and incubated with PBLs from 10 healthy CMV-seropositive donors. Targets included autologous BBs, BBs pulsed with pp65 and IE-1 overlapping peptides, and allogeneic BBs.

RESULTS

To determine whether CMV and IE-1–specific CTL could be regularly expanded from normal donors, we stimulated PBLs with a peptide mix derived from IE-1 and pp65 simultaneously. Immunophenotyping showed that the most of the cells were $CD3^+$, with a mixture of $CD4^+$ and $CD8^+$ effector cells also present, as described in Table 1. The cytotoxicity data (Figure 1) revealed a lack of autoreactivity or alloreactivity in these CTLs. Of the 10 donors tested, 4 had cytotoxicity to targets expressing both antigens, 4 had pp65-specific cytotoxicity only, 1 had IE-1 cytotoxicity only, and 1 responded to neither antigen. CRA data on CTLs cultured separately with either pp65 or IE-1 peptide-pulsed monocytes or with both peptide mixes combined show that the simultaneous use of these peptides did not compromise cytotoxicity in most donors (Table 2). To confirm that these CTLs recognized naturally processed pp65 and IE-1 epitopes, CRA also was performed using BBs infected with vaccinia encoding either pp65 or IE-1. The results, shown in Figure 2, indicate similar cytotoxicity against vaccinia pp65 and IE-1–infected BBs as was seen from the use of peptides, demonstrating that these CTLs recognized naturally processed and presented epitopes of both antigens.

Figure 3 illustrates cytokine production by 3 of our donors, 2 donors with cytotoxicity and cytokine production to only 1 of the antigens and 1 donor with IFN-γ production and cytotoxicity to both antigens. In general, the presence of cytotoxicity for either pp65 or IE-1 was correlated with $CD4^+$ and $CD8^+$ T cells producing IFN-γ in response to these antigens, as shown in Table 3. These donors had diverse HLA backgrounds and varying levels of response to these antigens. Donor 1 had IE-1–specific cytotoxicity and $CD4^+$ and $CD8^+$ cells producing IFN-γ in response to this antigen. This donor lacked pp65-specific cytotoxicity as well as $CD8^+$ IFN-γ–specific T cells, but 0.6% of the cells were IFN-γ producing $CD4^+$ pp65-specific T cells. Donor 3 had IFN-γ producing $CD8^+$ T cells specific for IE-1 but lacked significant cytotoxicity to this antigen. Donor 8, who was CMV-seropositive but lacked cytotoxicity to either antigen, did not have significant levels of IFN-γ–producing T cells to pp65 or IE-1. Therefore, although there seems to be a close correlation between IFN-γ production and cytotoxicity, as expected, some donors without cytotoxicity may have IFN-γ production in response to a specific antigen.

All CTL were cultured for 10 days, with a mean 1.8 ± 0.7-fold increase (range, 1.1 to 2.5) in the number of cells seen at the end of the culture period. Therefore, for a typical adult patient weighing 70 kg, a 100-mL blood draw from the stem cell donor permitted a cell

Table 1. Phenotype of CTLs stimulated with combined pp65 and IE1 (%)

	CD3	CD4	CD8	CD56
Donor 1	89	32	53	0.9
Donor 2	95	28	65	1.5
Donor 3	90	51	36	1.4
Donor 4	90	50	38	0.7
Donor 5	86	62	22	1.8
Donor 6	88	42	45	0.6
Donor 7	89	57	30	1
Donor 8	83	54	29	1
Donor 9	97	38	53	8.2
Donor 10	81	32	40	3

Table 2. Cytotoxicity of CTLs stimulated with pp65, IE-1 alone, and combined pp65 and IE-1 (%)

			Mixed CTL pp65 + IE-1	
	CTL pp65	CTL IE-1	pp65	IE-1
Donor 1	8	60	6	66
Donor 2	69	50	54	41
Donor 3	37	4	24	12
Donor 4	47	66	63	59
Donor 5	50	37	62	30
Donor 6	45	8	51	8
Donor 7	46	5	41	6
Donor 8	-1	-2	-3	-2
Donor 9	53	69	31	56
Donor 10	36	-1	31	3

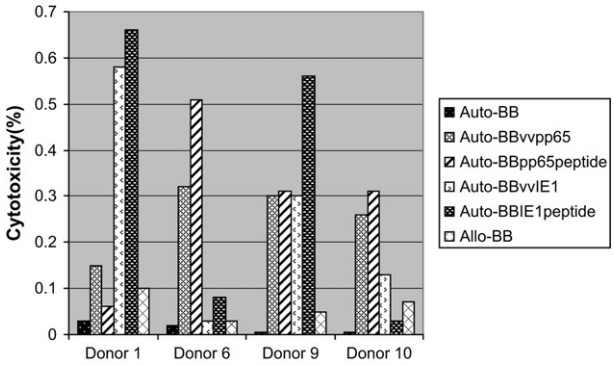

Figure 2. Cytotoxicity of CMV CTLs. PBLs were stimulated with autologous monocytes pulsed with pp65 and IE-1 overlapping peptides. Specific cytotoxicity was measured using BBs infected with either vaccinia encoding pp65 or IE-1 (vvp65, vvIE1), as well as BBs pulsed with either peptide mix. Autologous and allogeneic BBs were used as negative controls.

dose of 5×10^5 $CD3^+$ CTLs/kg. Peggs et al. [5] demonstrated that CTL doses as low as 1×10^5 cells/kg could result in CMV-specific immune reconstitution, although 2 of their 14 CTL recipients required a second infusion for subsequent CMV reactivation. Therefore, a cell dose of 2 to 5×10^5 $CD3^+$ CTLs/kg should be adequate for most patients. We compared the costs of preparing and shipping a single dose of 5×10^5 $CD3^+$ CTLs/kg (for a 70-kg patient) with the costs of maintenance therapy with antiviral agents (cidofovir, ganciclovir, valganciclovir, and foscarnet); the data are presented in Table 4. This cost analysis did not take into account the costs of laboratory monitoring, drug administration, and medications or fluids to treat or prevent complications. From the data, it appears that the costs of preparing, qualifying, and shipping CMV CTLs using our proposed method would be comparable to or less expensive than average wholesale prices for maintenance courses of these antiviral agents.

DISCUSSION

The decision to administer CMV CTLs must be based on an understanding of the risks associated with CMV infection; the time frame, feasibility, and cost of CTL culture; as well as the risks associated with administering CTLs. The method that we used to generate CMV-specific CTLs is simple and bypasses the requirement for gene therapy or the use of other techniques that may be available at only a few transplantation centers. Our method also is less expensive than other methods of CTL selection, such as cytokine capture. Because of the use of specialized reagents and labware, the cost of cytokine capture (including quality assurance testing of the final product) is more than threefold greater than that of our method, not including the expense of a cell selection device. Considering the relatively low cost, feasibility, and low risk for graft-versus-host disease (GVHD), cellular immunotherapy for CMV could be considered a reasonable option either at the time of reactivation or as prophylaxis in high-risk patients. As the cost analysis demonstrates, the cost of CTL preparation does not differ significantly from that of standard courses of antivirals, and the latter may need to be used over a prolonged period if viremia persists or recurs. Moreover, antiviral drugs can cause multiple side effects, most commonly nephrotoxicity and myelosuppression. These toxicities are compounded by the fact that many SCT recipients may have cytopenias, and most are receiving other nephrotoxic agents. The frequency of neutropenia ranges from 40% to 60% in SCT patients who receive ganciclovir [16,17]. Moreover, the use of ganciclovir has been associated with increases in bacterial sepsis and invasive fungal infections [16,18,19] and also has a negative effect on CMV-specific cellular immune responses as well as lymphocyte function in general [2]. The use of a second-line agent, such as foscarnet or cidofovir, can be limited by the potential for severe nephrotoxicity, necessitating intravenous fluid administration and careful monitoring of hydration and electrolyte status [20]. Intervention with CMV-specific CTLs not only results in virus-specific immune reconstitution and prevention of further viremia, but also could reduce overall patient care costs.

The primary motivation for expanding IE-1– and pp65-specific $CD4^+$ and $CD8^+$ T cells simultaneously is to provide CTLs with reactivity against viral epitopes that are relevant for a broad group of patients. Our work and other studies [21] has revealed a wide diversity in CD4 and CD8 T cell responses to different CMV antigens in normal donors. Variability in the relative numbers of CD4 and CD8 effector cells and IFN-γ production among normal donors could reflect individual CMV-specific cellular immunity, as well as differences in HLA background and epitope dominance. These variables will likely result in differences in the degree of T cell responsiveness to epitopes presented in the peptide mix, some of which may not be presented optimally. Previous studies have demonstrated the importance of CMV-specific $CD4^+$ T cells for achieving long-term immune reconstitution to this virus [22]. Our findings indicate that both $CD4^+$ and $CD8^+$ pp65- and IE-1–specific effector cells were present after stimulation with these peptide mixes, and that the ratios of CD4 and CD8 T cells were well balanced. The patterns of antigen-specific cytotoxicity were similar regardless of whether pp65 and IE-1 CTLs were stimulated separately or simultaneously. Although some donors respond to a given antigen but not to others, some individuals demonstrate IFN-γ responses to an antigen but lack activity in CRA. It is possible that with further courses of

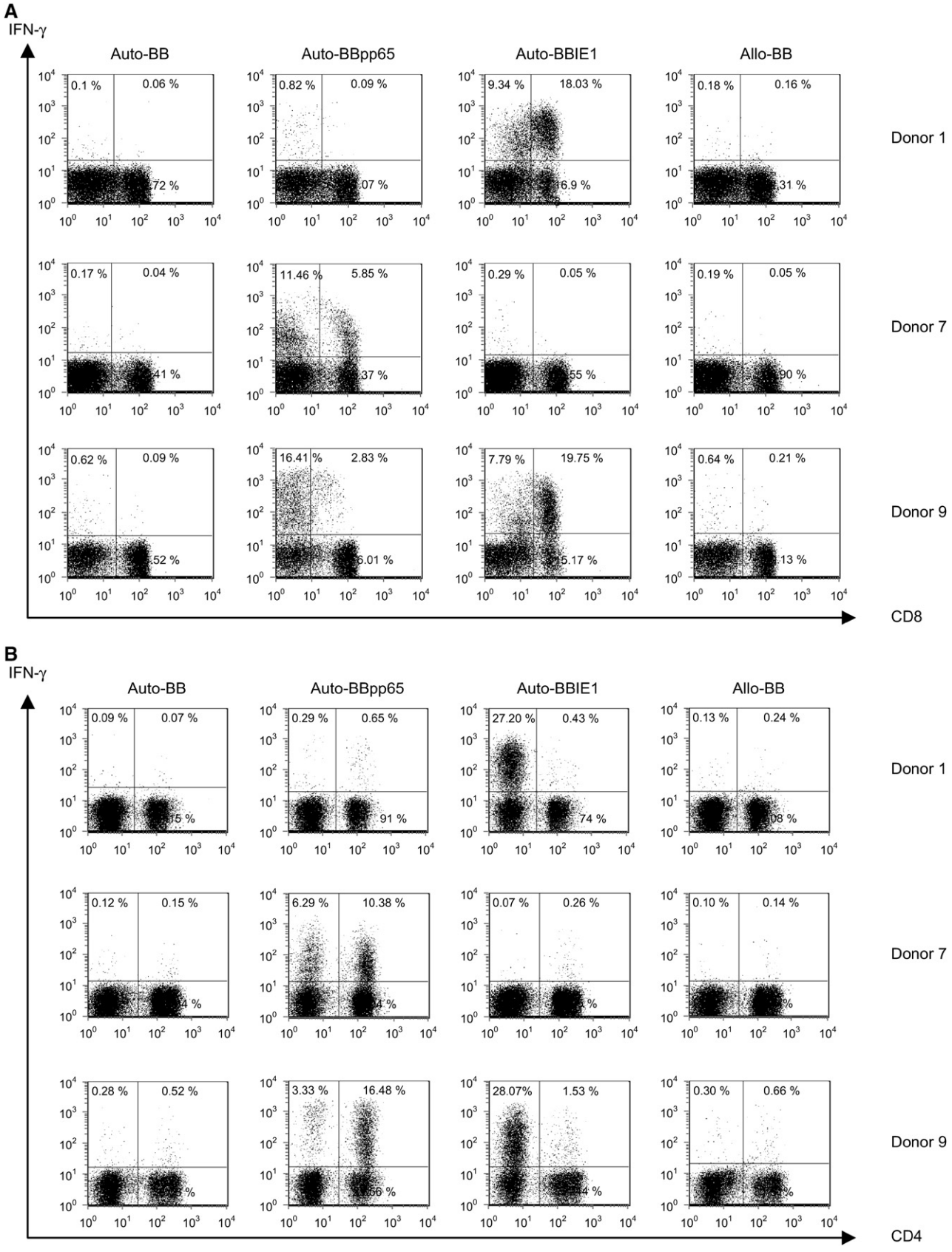

Figure 3. IFN-γ production of T cells. PBMCs were pulsed with pooled CMV pp65 peptides and IE-1 peptides and incubated with nonadherent PBLs. IFN-γ producing T cells specific for these CMV peptides were analyzed at day 10 of culture by intracellular staining. A, $CD8^+$ T cell response to CMV pooled peptides. B, $CD4^+$ T cell response to the CMV peptides.

Table 3. IFN-γ production by CTL stimulated with combined pp65 and IE-1

	Response to pp65 (%)		Response to IE-1 (%)		HLA typing		
	CD4	CD8	CD4	CD8	HLA- A	HLA- B	HLA-DR
Donor 1	0.59	0.09	0.38	23	11	38, 54	04, 08
Donor 2	0.98	19.08	0.39	3.96	02, 03	07, 55	04, 14
Donor 3	0.81	2.42	0.12	0.44	11, 24	07, 51	01, 03
Donor 4	2.2	5.3	0.36	6	02	27, 41	01, 08
Donor 5	10.86	7.3	0.8	0.78	02, 26	41, 44	08, 11
Donor 6	0.48	5.31	0.26	0.91	02, 26	46, 48	08
Donor 7	10.4	5.25	0.26	0.02	01, 02	13, 60	07, 11
Donor 8	0.17	0.04	0.15	0.05	02, 25	18, 60	01, 04
Donor 9	16.48	1.82	1.53	21.61	11, 24	54, 60	14, 15
Donor 10	0.71	2.81	0.13	0.19	11, 31	35, 60	04, 09

stimulation, these donors could develop cytotoxicity. Further study is needed to determine the role of cytokine-producing effector cells for donors who lack cytotoxicity to a specific antigen.

Although CMV pp65 has been shown to be the immunodominant CMV antigen, IE-1–specific immunity also is important in protecting against CMV [11]. In organ transplant recipients, Bunde et al. [23] demonstrated that having high frequencies of IE-1–specific T cells in the early posttransplantation course was protective against developing CMV disease. Khan et al. [11] reported that IE-1–specific T cells increase over time postinfection and may be effective in preventing low-level viral reactivation after an acute infection. Other studies have shown that a situation of a higher proportion of pp65-specific T cells than IE-1–specific T cells makes IFN-γ and tumor necrosis factor-α and has greater cytotoxicity in SCT and organ transplant recipients [24]. Studies are currently underway at our center and others examining the use of CMV vaccines in seronegative stem cell donors. One such vaccine consists of the Towne strain of CMV, which has been shown to result in higher levels of IFN-γ–producing $CD8^+$ T cells to IE-1 compared with pp65 [13]. Whereas pp65 responses decrease over time in these vaccine recipients, IE-1 responses tend to be maintained; thus, CTLs with reactivity to more than 1 antigen is of potential benefit. Although priming T cells with CMV lysate could result in cells with a broader range of specificity, this method of stimulation has been shown to favor $CD4^+$ T cell expansion [4].

Table 4. Cost of Antiviral Agents and CTL

Regimen, for a 70-kg patient, 28 days	Average wholesale price (USD)*
Ganciclovir, 5 mg/kg/day i.v.	1048.60
Foscarnet, 90 mg/kg/day i.v.	1234.80
Valganciclovir, 15 mg/kg/day p.o.	1915.20
Cidofovir, 5 mg/kg/week every 2 weeks	1657.60
Single infusion of CMV CTLs	1350.00

*These figures include quality assurance testing, cryopreservation, and shipping of CTLs but do not account for intravenous administration costs, compounding costs of oral medications, charges for laboratory monitoring, or costs of medications to treat or prevent drug-related complications. Drug costs are from Cardinal Health Corporation, April 2008 (http://www.cardinal.com).

An overriding concern with any use of cellular immunotherapy after allogeneic SCT is the efficacy of the product and the risks for GVHD, although this complication generally is not seen with adoptive immunotherapy using virus-specific CTLs [4,7]. The cellular products described in this report lack autoreactivity or alloreactivity in CRA, and 2 patients who have received pp65-specific CTL using this method have not developed GVHD as of the time of this writing (in press). With selective expansion of CTLs, we would expect to see a decrease in potentially alloreactive CTLs and an enrichment of CMV CTLs over the 10 days in culture, and the administration of relatively low cell doses as in our current protocol (2 to 5×10^5 $CD3^+$ CTLs/kg) likely would decrease the risk of GVHD. Situations in which there is no urgency for culturing CTLs (as when cells are cultured prospectively for prophylaxis) would allow for longer culture times, further reducing the risk of GVHD. The minimal cell dose needed for immune reconstitution using CMV-specific CTLs has not yet been defined; it will depend on the level of enrichment of virus-specific cells. MacKinnon et al. [25] reported the use 1×10^5 $CD3^+$ CMV- specific T cells/kg, with no recipients developing GVHD and most recipients experiencing no subsequent CMV reactivation. This group also described the selection of CTLs based on IFN–γ capture [9], with a mean CMV-reactive T cell dose of 3.4×10^6 total cells per patient. The administration of lower doses of CMV-specific T cells in the future would reduce the GVHD risk and permit the cryopreservation of several vials for subsequent infusions, if needed.

The use of both CMV IE-1 and pp65 peptides covers immunologically relevant CMV antigens for most individuals, although some donors may not respond to either antigen after 10 days of culture and may need subsequent stimulation with these peptides. For those at high risk for CMV reactivation, prospectively culturing these CTLs from seropositive donors could be considered, with the CTLs infused either

prophylactically or at reactivation. With several studies demonstrating the efficacy of adoptive immunotherapy with CMV CTLs, and with a practical method whereby these cells can be expanded, this form of therapy could be considered for more patients, particularly in light of the high cost and deleterious side effects of most antiviral agents. It also is possible that CTLs with specificity to other viral, fungal, or tumor antigens could be cultured concurrently using this method, as has been described previously using gene therapy approaches. We will be implementing this new strategy to stimulate donor-derived IE-1 and pp65 CTLs for SCT patients with CMV reactivation, including those with donors who have received a CMV vaccine.

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